The Specific Inhibition of the Cardiac Electrogenic Sodium/Bicarbonate Cotransporter Leads to Cardiac Hypertrophy.

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Abstract

Two alkalinizing mechanisms coexist in cardiac myocytes in order to maintain the intracellular pH: sodium/bicarbonate cotransporter (electroneutral isoform NBCn1 and electrogenic isoform NBCe1) and sodium/proton exchanger (NHE1). The dysfunction of these transporters has been previously reported as responsible for the development of cardiovascular diseases. The aim of this work was to evaluate the contribution of the downregulation of the NBCe1 to the development of cardiac hypertrophy. To specifically reduce NBCe1, we used shRNA cloned into a cardiotropic adeno-associated vector (AAV9-shNBCe1). After 28 days of being injected with AAV9-shNBCe1, the expression and the activity of NBCe1 in the rat heart were reduced. Strikingly, the downregulation of NBCe1 causes significant hypertrophic heart growth, lengthening of the action potential in isolated myocytes, increase in the duration of the QT interval and increase in the frequency of Ca^{2+} waves without any significant changes in Ca^{2+} transients. An increased compensatory expression of NBCn1 and NHE1 was also found. We conclude that the reduction of NBCe1 is sufficient to induce cardiac hypertrophy and modify electrical features of the rat heart.

Introduction

Despite the extraordinary efforts of the scientific and medical community, cardiovascular disease (CVD) is still the main cause of death globally, with more than 17 million of deaths per year, representing more than 30% of all global deaths ^{1,2}. Particularly, cardiac hypertrophy (CH) is one of the main factors for CVD in individuals with hypertension. CH is characterized by abnormal thickening of the left ventricle wall and implies the maladaptive compensatory response to a sustained high blood pressure or a cardiac insult. Furthermore, this ventricular enlargement is accompanied with fibrosis and metabolic changes and, in case of persisting through time, it could progress to heart failure and malignant arrhythmia ^{3,4}.

Intracellular pH (pH_i) maintenance is required for any physiological process. Specifically, in cardiomyocytes is crucial for the correct function of many key features, such as electrical activity and contractility, which cannot be guaranteed under acidic conditions ⁵. Thus, cardiac cells rely on two sarcolemmal alkalinizing transporters: Na^+/H^+ exchanger or NHE1, which catalyses the influx of one Na^+ per each H^+ removed from the cytosolic space, and the $\text{Na}^+/\text{HCO}_3^-$ cotransporter or NBC, which drives the co-influx of Na^+ and HCO_3^- . There are currently two known cardiac isoforms of NBC, an electroneutral (NBCn1 of stoichiometry $1\text{Na}^+:1\text{HCO}_3^-$) and an electrogenic one (NBCe1 of stoichiometry $1\text{Na}^+:2\text{HCO}_3^-$) ⁶.

Unlike NHE1, both cardiac NBC isoforms are localized in t-tubules of myocytes, suggesting an important role in the local pH regulation of the dyadic space ^{7,8}. An alteration of the pH in these microdomains could affect the function of Ca^{2+} handling proteins (RYR, SERCA, PLN, NCX), leading to a deregulation of Ca^{2+} homeostasis.

Another important aspect of cardiac NBC isoforms are their electrical properties. The movement of one negative charge inside the cell through the NBCe1 generates a repolarizing current described as an anionic bicarbonate and sodium-dependent current which reverses at around -85mV (I_{NBC}). Moreover, I_{NBC} impacts on the configuration of the cardiac action potential (CAP) of ventricular myocytes^{9,10}.

There is compelling evidence that over-activation of NHE1 leads to elevated intracellular Na^+ concentration $[\text{Na}^+]_i$, increasing Ca^{2+} influx through reduce Ca^{2+} extrusion via forward mode $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) or even could activate the reverse mode of the NCX¹¹⁻¹⁴. This mechanism could promote the activation of the calcineurin/NFAT pathway and contribute to cardiac hypertrophy development¹⁵. Since NBC also promotes Na^+ uptake, it is reasonable to hypothesize the same mechanism as previously proposed for NHE1. However, each NBC isoform would contribute differently to Na^+ uptake. Due to its stoichiometry, NBCn1 contributes one Na^+ per HCO_3^- in each transport cycle, whilst NBCe1 uses only half of Na^+ per HCO_3^- . This differential contribution of Na^+ could be relevant to the role of each isoform in the development of CH.

Our and other laboratories have demonstrated a differential activity and/or expression of cardiac NBC isoform in different *in vivo* hypertrophy models¹⁶⁻¹⁹. Moreover, we showed that the activity of NBCe1 is decreased while NBCn1 is over-expressed and over-activated in the hypertrophied myocytes of spontaneous hypertensive rats (SHR)¹⁷. However, whether the decrease in NBCe1 activity was a cause or consequence of the development of CH have not been confirmed yet.

In the present work, we attempted to respond to this question by designing and developing a shRNA against NBCe1. The shNBCe1 was cloned in a recombinant adeno-associated viral vector (AAV9-shNBCe1) in order to specifically inhibit NBCe1 in rat hearts. Here, we demonstrated that the downregulation of cardiac NBCe1 is sufficient to induce CH.

Material And Methods

All experiments were performed in accordance to the Guide for Care and Use of Laboratory Animal and approved by the Ethics committee of the Faculty of Medicine, La Plata, Argentina Institutional Animal Care and Use Committee (CICUAL no. P03-01-2017) and adhered to the ARRIVE guidelines. All experiment procedures and evaluations were performed in a blinded manner.

Myocytes isolation

Ventricular myocytes were isolated according to the technique previously described,²⁰ using 4-month-old Wistar rats.

AAV9-shNBCe1 and AAV9-shControl production

Recombinant AAV9-shNBCe1 or AAV9-shControl was produced essentially as previously described (Orlowski et al., 2021 and Lopez-Gordo et al., 2019)^{21,22}. Briefly, HEK293T/17 (ATCC) cells were cultured with DMEM (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Natocor) and penicillin/streptomycin (Thermo Fisher Scientific) at 37°C and 5% CO₂. Once 90% confluence was reached, transfection was performed by adding cis-plasmid (shNBCe1: GGACCAGCTGAAGAATAAATT and shControl: CCTAAGGTTAAGTCGCCCTCG were construct by Vector Builder®), pDG9 (gift from Roger Hajjar) and PEI-max (Polysciences) solution (pH 4.5). After 3 days of transfection, cells and supernatant were harvested. The cell pellet was resuspended in lysis buffer (150 mM of NaCl, 50 mM of Tris hydrochloride, pH 8.5, and 2 mM of MgCl₂), and freeze-thawed three times at -80°C. Supernatant was precipitated with 31.3 g of ammonium sulphate per 100 mL and incubated on ice for 30 min. After the incubation the supernatant was centrifuged at 8300 x g (avg) for 30 min at 4°C. The precipitate was resuspended in lysis buffer and combined with cell lysate. Pierce Universal Nuclease (Thermo Fisher Scientific) was used to digest non-encapsidated DNA and RNA. After 30 min of incubation the virus contain solution was centrifuged at 8000 x g (avg) for 30 min and the precipitate was discarded. For virus purification a discontinuous iodixanol gradients (4 mL of 60% iodixanol, 4 mL of 40%, 4.9 mL of 25%, and 7.3 mL of 15% (with 1 M sodium chloride)) were prepared on polypropylene Optiseal tubes (Beckman Coulter). The gradients were centrifuged at 350,333 × g (avg) for 75 min at 18°C in a Beckman type 70 Ti fixed angle rotor. Fractions were obtained from the bottom of the tube and the presence of the virus were detected by alkaline gel²². Positive fractions were buffer exchange lactated Ringer's solution (Baxter International), filtered through a 0.22 µm pore filter (Merck Millipore), and stored at -80°C.

Administration of AAV9

A tail vein injection of the adeno-associated vector was performed (1×10^{12} vector genomes/ml per rat). The animals were anaesthetized with an inhalant anaesthetic (isoflurane) and the injection was performed with a 27.5-gauge.

Echocardiographic study

Rats were monitored echocardiographically at the start and end of the treatment, under light anaesthesia with isoflurane by 2-dimensional and M-mode echocardiography with a 14-MHz transducer (Toshiba Nemio XG). Measurements were performed according to the method proposed by the American Society of Echocardiography.

Electrocardiographic (ECG) study

Surface ECGs were acquired at 40 KHz sampling rate using standard ECG electrodes (lead I) for the PowerLab 4ST data acquisition system (Adinstruments) as previously described²³ with modifications. Rats were anaesthetized with an inhalant anaesthetic (isoflurane) during the procedure. Recordings were performed for two minutes by inserting the electrodes on the skin of the rats, and after verifying a stable heart rate. For measuring QT interval, approximately 50 ECG traces were averaged. QT corrected (QTc)

values were obtained from the Bazett formula: $QTc = QT/(RR)^{1/2}$, wherein RR is the averaged period between the R waves of two consecutive traces.

Cross sectional area measurement

Cross sectional area measurement were performed as previously describe²⁴. Briefly, ventricular tissue was fixed in buffered 10% formaldehyde and paraffin embedded. LV sections (5 µm thick) at the equator were stained with hematoxylin-eosin for determining cardiomyocyte cross-sectional area (CSA). To assess CSA, only round to ovoid cells with visible round nucleus were considered, and 50 cells were counted in at least 10 images obtained from each left ventricle. Each cell was individually traced and its cross-sectional area directly determined. All the stained sections were observed under the microscope (Olympus BX-53) and the images were captured using a digital video camera (Olympus DP-71). Images were digitized and processed by a computer software (Image-Pro Plus®). The investigator responsible for the morphological analysis was blinded as to each experimental group.

Western blot analysis

Ventricular protein expression by western-blot were essential done and describe earlier in more details²⁴. The primary antibodies used were anti-loop 3, anti-NBCe1 produced in our laboratory⁶ (1:500), anti-NBCn1 (Santa Cruz Biotechnology, 1:1000), anti-NHE1 (Santa Cruz Biotechnology, 1:1000) or anti-GAPDH (Ambion, 1:1,000). Peroxidase conjugated anti-rabbit (Santa Cruz Biotechnology, 1:10,000) or m-IgGκ BP (Santa Cruz Biotechnology, 1:10,000) were used as secondary antibodies. Chemiluminescence detection (Amersham, ECL-Plus) and Chemidoc Image Station (Bio-Rad) was used to visualize the blots and they quantified by densitometric analysis (Image J Fiji®).

Real time qPCR

Quantitative PCR was performed as describe before²⁴. RNA was extracted from hearts using TRIzol reagent (Life Technologies). cDNA was generated by reverse transcriptase reaction using M-MLV RT (Promega). Real time quantitative PCR was performed on cDNA using the IQ SYBR green SuperMix (Bio-Rad) and iCycler iQ (Bio-Rad). The following primers were used:

ANP Gene Sense: GGATTTCAAGAACCTGCTAGACCA, Antisense: CTGCTTCCTCAGTCTGCTCACTCA

GAPDH Gene Sense: GGGTGTGAACCACGAGAAAT, Antisense: CCACAGTCTTCTGAGTGGCA.

Relative abundance of RNA was calculated by the $\Delta\Delta C_t$ method. Primers were designed using

Primer-Blast (NCBI, NIH). All primers were 90–110% efficient, as assessed by standard curve, and all displayed only 1 dissociation peak.

Patch-clamp recordings

Action potentials (APs) were recorded with the nystatin perforated whole-cell configuration of the Patch Clamp technique using current-clamp recording as previously describe²⁵ with some modifications. Axopatch 200B amplifier and analog-to-digital converter Digidata 1322A (Molecular Devices) were used to acquire APs recorded with pClamp 9.2 Software (Molecular Devices). Borosilicate patch pipettes were pulled with P-97 puller (Sutter Instruments) to a final resistance of 2 - 3 MΩ. The pipette was filled with (mM): 125 K-Gluconate, 10 KCl, 8 Na-Gluconate, 1 MgCl₂, 10 HEPES and 0.30 mg/ml of Nystatin (pH 7.2 with KOH, final concentration of K⁺: 140 mM). The HCO₃⁻ solution contained (mM): 120 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 20 NaHCO₃, 5 Glucose and 13 Choline-Cl (pH 7.4 with 95% O₂ - 5% CO₂). APs were triggered by 2 ms squared pulses of depolarizing current at 1 Hz. All data recorded was compensated with the Junction Potential of -14.7 mV in the HCO₃⁻ solution (Junction Potential was calculated with the Junction Potential Tools of pClamp Clampex 10.3 Software). The Patch Clamp data was processed with ClampFit 10.3 (Molecular Devices) and analysed in GraphPad Prism 8. All experiments were performed at room temperature (22-25°C).

pH_i measurement

Intracellular pH measurement were performed in rat isolated ventricular cardiac myocytes and the detail protocol was detailed in previously works^{8,26}. Cells were incubated with 10 μM BCECF-AM followed for 10 min and placed in a chamber on the stage of an inverted microscope (Nikon TE 2000-U) couple to an epi-fluorescence system (Ion Optix). The chamber was superfused with a solution containing (mM) 5 KCl, 118 NaCl, 1.2 MgSO₄, 0.8 Cl₂Mg, 1.35 Cl₂Ca, 10 glucose, 20 NaHCO₃, pH 7.4 after continuous bubbling with 5% CO₂ and 95% O₂. Myocytes were field stimulated by two-platinum electrodes on either side of the bath at 0.5 Hz. At the end of each experiment, the fluorescence ratio was converted to pH the high K⁺-nigericin method⁶.

Potassium pulse

To investigate the NBCe1 activity in isolation we performed a potassium pulse^{6,26}. The high K⁺ solution ((mM): 118 NaCl, 5 KCl, 1 MgSO₄, 0.35 NaH₂PO₄, 10 glucose, 40 KCl, 20 NaHCO₃, pH 7.4) was applied for 14 minutes and during this period the pH_i was recorded. The data were expressed as an increase of pH_i units in comparison to the zero time point in high K⁺ solution.

Detection of cytosolic Ca²⁺ by confocal microscopy

Cardiomyocytes were loaded with 10 μM Fluo-4 AM (Thermo fisher scientific), dissolved in 20% F-127 pluronic in dimethylsulfoxide (DMSO). After 10 minutes of loading at room temperature, cells were washed and seeded on a perfusion chamber. The chamber was mounted on a Zeiss 410 inverted confocal microscope (LSM Tech) equipped with a 63x, 1.4 NA oil immersion objective. All experiments were performed at room temperature (24°C). Myocytes were repetitively field stimulated with 10-ms duration square wave pulses delivered via platinum wires. Fluorescence excitation was achieved with a

488 nm argon laser, and 500-550 nm emitted light was collected. Fluo-4 fluorescence was recorded in linescan mode, with a 512 pixels line placed along the long axis of the cell. Each recording consisted of 512 consecutive linescans (4.3 msec per line), which were stacked to create space-time profiles. In order to measure spontaneous Ca^{2+} release, pacing frequency was enhanced to 5 hz for a minute and then stopped to quantify the occurrence of spontaneous Ca^{2+} waves.

Statistics

Statistical analyses were performed using version 8 of Prism (GraphPad) and R version 4.1.2. All the data sets were analysed with Shapiro-Wilk normality test. Therefore, group-to-group comparisons were performed using t-Student (normal distribution) or Mann-Whitney test (not normal distribution). Data were expressed in the text as means $\pm$ S.E.M. Data in the figures were expressed as box and whisker plot. A value of $P < 0.05$ was considered statistically significant (two-tailed test). The investigator responsible for each experiment were blinded as to each experimental group.

Results

In vivo downregulation of NBCe1 by AAV9-shRNA

Recombinant adeno-associated virus 9 (AAV9) are the leading tool for *in vivo* heart transduction of short hairpin RNA (shRNA), since AAV9 produces high myocardium transduction and long-term transgene expression¹⁹. In order to downregulate cardiac NBCe1, we generated two cardiotropic AAV9 vectors encoding the enhanced green fluorescent protein (eGFP) under CMV promoter and shRNA against NBCe1 (shNBCe1) or shLacZ (shControl) (**Fig. 1a**). Specific knockdown of rat NBCe1 was carried out by systemic tail vein injection of AAV9-shNBCe1. **Fig. 1b** shows the experiment design; *in vivo* heart parameters (echocardiography, electrocardiogram and blood pressure) were follow-up during the experiment. After 28 days of AAV9 injection, cardiac myocytes were isolated or heart tissue was collected. Myocyte's transduction was evidenced by the expression of eGFP protein in heart tissue sections or in isolated myocytes (**Fig. 1c**). NBCe1 was significantly reduced in total hearts homogenates of rats injected with AAV9-shNBCe1 (shControl: 1.00 ± 0.11 , $n=7$; shNBCe1*: 0.71 ± 0.04 , $n=5$; * $p < 0.05$ vs shControl) (**Fig. 1d**). Voltage-dependence NBCe1 activity was tested in isolated rat cardiac myocytes after 28 days of AAV9 injection (**Fig. 1e**). Cells were loaded with a fluorescent pH indicator (BCECF) and superfused with hyperkalemic isosmotic solution, intracellular pH response was monitored during the protocol. Depolarization of the cell increased NBCe1 activity visualized by an alkalinisation of intracellular pH. Despite the moderate decrease in protein expression, isolated myocytes from shNBCe1 injected rats present a significantly reduced NBCe1 activity compared with the shControl rats (shControl: 0.15 ± 0.05 , $n=5$ myocytes from 3 rats; shNBCe1*: 0.03 ± 0.02 , $n=7$ myocytes from 3 rats; * $p < 0.05$ vs. shControl) (**Fig. 1f**). These results indicated that a single AAV9-shNBCe1 injection reduced NBCe1 expression and consequently its activity.

Downregulation of NBCe1 induces the development of cardiac hypertrophy

Cardiac hypertrophy was previously associated with the activity of NHE1 and NBC. While an increase in NHE1 activity is accepted to induce cardiac hypertrophy, for NBC it seems to depend on the isoform involved. To determine if a reduction of NBCe1 activity plays a role on cardiac hypertrophy development, rats were followed-up by echocardiography before and after AAV9 injection. Strikingly, left ventricular mass index and wall thickness was increased in shNBCe1 rats (**Fig. 2a and 2b**) and it was independent of blood pressure (**Fig. 2c**). Cardiac function remained without significant changes when fractional shortening (shControl: 0.91 ± 0.11 , $n=12$; shNBCe1: 0.82 ± 0.08 , $n=11$) and ejection fraction were measured (shControl: 0.96 ± 0.05 , $n=12$; shNBCe1: 0.91 ± 0.04 , $n=11$). After 28 days of AAV9 injection, hearts were collected and left ventricular sections were used for determining myocytes cross sectional area (CSA). Consistent with echocardiography results, shNBCe1 CSA was significantly higher compared to shControl (μm^2 ; shControl: 247.5 ± 13.7 , $n=9$; shNBCe1*: 297.6 ± 15.9 , $n=8$; * $p < 0.05$ vs shControl) (**Fig. 2d**). Nppa (ANP) mRNA levels were assessed as a hypertrophic marker. shNBCe1 hearts showed an increase of the levels of ANP when it was compared to the shControl group (shControl: 1.00 ± 0.24 , $n=5$; shNBCe1*: 2.18 ± 0.44 , $n=5$; * $p < 0.05$ vs. shControl) (**Fig. 2e**). Taken together, these findings indicate that the silencing of NBCe1 is enough to develop cardiac hypertrophy in rats.

NBCe1 influences the electrical properties of rat hearts.

Due to its electrical properties NBCe1 contributes to the duration of the action potential (APD) of cardiac myocytes. An increase of its activity shortens the action potential and hyperpolarizes the resting membrane potential (RMP). On the contrary, a decrease of NBCe1 activity prolongs the APD and depolarizes the RMP. Taking this into account, we evaluated the APD and RMP in rat isolated cardiac myocytes previously injected with shNBCe1 or shControl. Isolated cardiomyocytes were infused with a bicarbonate buffer and APD and RMP were measured employing perforated patch clamp technique (**Fig. 3**). We found that the RMP of cardiomyocytes from the shNBCe1 group were depolarized in comparison to the shControl group (RMP (mV): shControl: -80.18 ± 0.51 , $n=12$ myocytes from 5 rats; shNBCe1*: -73.82 ± 2.18 , $n=12$ myocytes from 4 rats; * $p < 0.05$ vs. shControl) (**Fig. 3b**). We studied the APD at 50% of repolarization time (APD₅₀) and we found a significant higher value in shNBCe1 group than shControl group (ms; shControl: 45.21 ± 4.74 , $n=10$ myocytes from 5 rats; shNBCe1*: 75.06 ± 14.30 , $n=9$ myocytes from 4 rats; * $p < 0.05$ vs. shControl) (**Fig. 3c**). Moreover, not significant differences were observed in RMP and APD₅₀ when HCO_3^- was removed from the buffer solution (RMP (mV): shControl: -80.3 ± 0.72 , $n=10$ myocytes from 5 rats; shNBCe1: -77.73 ± 2.18 , $n=4$ myocytes from 4 rats; APD₅₀ (ms): shControl: 70.60 ± 5.86 , $n=8$ myocytes from 5 rats; shNBCe1: 76.07 ± 7.44 , $n=6$ myocytes from 4 rats), indicating that the effects of NBCe1 silencing are bicarbonate-sensitive. These results support that the reduction of NBCe1 activity affects the electrical properties of isolated rat ventricular myocytes.

To determine if the electrical changes found in isolated cells are extended to the entire heart, we performed rat electrocardiogram evaluation (ECG). **Fig. 4a** shows representative traces of ECG of rats before and after 28 days of shNBCe1 or shControl treatment. The QT interval was measured in order to evaluate the contribution of NBCe1 activity. Consistent with patch clamp experiments, we observed a

significant frequency corrected QT interval (QTc) prolongation in rats with a decrease of NBCe1 activity (**Fig. 4b**). The heart rate remained unchanged throughout the treatment (**Fig. 4c**).

Downregulation of NBCe1 is compensated by NBCn1 and NHE1 upregulation

In order to study if the reduction of NBCe1 protein and activity may be compensated by overexpression of other alkalinizing mechanisms, like NBCn1 and/or NHE1, western-blot experiments were performed (**Fig. 5**). Differential expression of NBC isoform has been implicated in CH, while NBCn1 are overexpressed, NBCe1 is diminished in different CH models. Moreover, it is widely known that NHE1 overexpression contributes to cardiac hypertrophy development and its inhibition prevents and regresses it. Here we found that the reduction of NBCe1 caused an increase of NBCn1 protein content (NBCn1: shControl: 1.00 ± 0.09 , n=5; shNBCe1*: 1.47 ± 0.15 , n=5; *p<0.05 vs. shControl) (**Fig. 5a**). NHE1 protein was also significant increased (NHE1: shControl: 1.00 ± 0.14 , n=6; shNBCe1*: 1.91 ± 0.32 , n=5; *p<0.05 vs shControl) (**Fig. 5b**). These results indicate that the overexpression of NBCn1 and NHE1 may act as a compensatory mechanism due to the reduction of NBCe1.

Increase in diastolic calcium waves in AAV9-shNBCe1 cardiomyocytes

Previous reports have shown alteration in Ca^{2+} handling in different models of cardiac hypertrophy in rodents ²⁷. With the purpose to study whether the lack of NBCe1 exhibit alterations in Ca^{2+} handling, isolated cardiomyocytes from rats injected with AAV9-shNBCe1 or AAV9-shControl were loaded with the intracellular Ca^{2+} indicator Fluo-4 and confocal line scans were performed. As can be observed in the representative images of Ca^{2+} transient (**Fig. 6a**) and in the average results (**Fig. 6b**), no significant differences were found between cardiomyocytes of AAV9-shNBCe1 and AAV9-shControl. We next examined spontaneous sarcoplasmic reticulum Ca^{2+} release by monitoring Ca^{2+} wave frequency as an indicator of Ca^{2+} mishandling (**Fig. 6c**). AAV9-shNBCe1 myocytes have significantly enhanced Ca^{2+} wave frequency compared with AAV9-shControl cells (**Fig. 6d**) (shControl: 0.020 ± 0.013 , n=10 myocytes from 2 rats; shNBCe1: 0.172 ± 0.036 , n=15 myocytes from 4 rats; *p<0.05 vs. shControl). Collectively, these results suggest that spontaneous SR Ca^{2+} release is enhanced in myocytes in which NBCe1 was downregulated.

Discussion

The aim of this work was to evaluate the contribution of the electrogenic isoform of cardiac sodium/bicarbonate cotransporter NBCe1 to the development of cardiac hypertrophy. To answer this question, we designed and developed a shRNA against rat NBCe1 cloned into a cardiotropic adeno-associated vector (AAV9-shNBCe1) to specifically reduce NBCe1 expression in rat cardiomyocytes. After 28 days of systemic AAV9-shNBCe1 tail injection, we found that the downregulation of NBCe1 causes significant hypertrophic heart growth compared to its control (AAV9-shControl) (**Fig. 7**). In addition, we described modifications in the electrical features of the heart. Through the patch clamp technique, we found a lengthening of the action potential in isolated myocytes transduced with shNBCe1. Interestingly, we showed for first time an increase in the duration of the QT interval indicating an *in vivo* impact of I_{NBC} .

The reduction in the expression of NBCe1 appears to be counterbalanced by other alkalizing transporters such as NHE1 and NBCn1 (**Fig. 7**). Finally, in isolated ventricular myocytes the downregulation of NBCe1 showed an increase in the frequency of Ca^{2+} waves without any significant changes in Ca^{2+} transients.

An increase in intracellular Ca^{2+} is related to the activation of prohypertrophic pathways such as calcineurin/NFAT¹⁵. It has been shown that hyperactivation of the NHE1 increases the intracellular Na^+ , leading to a rise in Ca^{2+} due to the deceleration of the forward mode of the NCX or even the activation of the reverse mode (rNCX)²⁸. As a result of this mechanism, a cytosolic increase of Ca^{2+} occurs in cardiac myocytes. A similar process could take place for NBC, since it is one of the major Na^+ -entry mechanisms of cells²⁹. In recent years, several works have described a relationship between NBC and the development of cardiac hypertrophy. Previous studies have shown that both NBC isoforms are overexpressed in cardiac hypertrophied rats with abdominal aortic constriction¹⁶. Furthermore, our laboratory also described an overexpression of both NBC isoforms in spontaneously hypertensive rats (SHR) that develops cardiac hypertrophy. In that opportunity, we found that the NBCe1 activity was impaired and compensated by an increase in NBCn1 activity¹⁷. Consistent with these results, *Ibañez et al*¹⁸, demonstrated in a model of ovariectomized rats, the increase in the activity of NBCn1 and a decrease in NBCe1. Recently, *Loonat et al*¹⁹, reduced epinephrine-induced hypertrophy using S0859, an NBC inhibitor³⁰, in neonatal rat cardiac myocytes. Unfortunately, this pharmacological inhibitor does not differentiate isoforms and therefore the contribution of each one to the development of cardiac hypertrophy was difficult to determine. Moreover, it has been described an up-regulation of NHE 1 mRNA in the heart of NBCe1 knockout mice³¹. In that work they showed the reduction of the apoptosis following *in vivo* ischemia-reperfusion injury, however they did not show the *in vivo* impact of NBCe1 in the development of cardiac hypertrophy.

In this work we demonstrated for the first time a causal relationship between the decrease in NBCe1 and the development of cardiac hypertrophy in normotensive rat hearts, which is independent of systolic blood pressure. A possible proposed mechanism of this phenomenon is a Na^+ overload due to compensation by NBCn1 and NHE1 which, unlike NBCe1, enter more Na^+ per alkaline equivalent. However, a modification of Na^+ or pH in the dyadic space, where NBCs are particularly expressed^{7,8}, cannot be ruled out. The regulation of these ions in this microdomain may be of utmost importance due to their differential function in the myocyte. Additionally, in this work we show that the decrease in the expression of NBCe1 causes an increase in the frequency of waves, which suggests a possible deregulation of Ca^{2+} handling in this microdomain.

Interesting results were described in a mice physiological hypertrophy model²⁵. Medina et al, showed that in mice that run on the wheel under their will, develop physiological hypertrophy that, unlike the pathological one, exhibited an increase in the activity of NBCe1. On the contrary, in transgenic mice that overexpress NBCe1, a decrease in NBCn1 was observed³². In the same work, they described an increase in intracellular Ca^{2+} concentration and the concomitant activation of deleterious pathways³².

The contradictions found in the literature regarding the role played by NBC isoforms in the development of cardiac hypertrophy reveal the complexity of this disease. In this study, for the first time, we were able to directly associate the decrease in NBCe1 and the concomitant increase in NBCn1 and NHE1 as the causal agent of the disease. However, it is possible that there are no pro or anti hypertrophic transporters, but rather the change in their activity contribution cause a modification of the homeostasis of the ions involved (H^+ , Na^+ and Ca^{2+}), generating the activation of the deleterious pathways in cardiac myocytes. Further research would need to be done to elucidate the responsibility of the NBCn1 in the development of cardiac hypertrophy.

A limitation of our work is that the expression of NBCe1 was not totally diminished. However, the activity was totally inhibited. A possible explanation for this discrepancy could be the cardiac tropism of AAV9. It is known that serotype 9 does not efficiently transduce fibroblasts and other cell types present in the heart³³, therefore it is possible that the expression of NBCe1 is the result of the sum of transduced and non-transduced cells. In addition, a similar strategy for NHE1 inhibition showed comparable results as obtained in this work^{34,35}.

Our laboratory has demonstrated the existence of the current generated by NBCe1 in rat and cat cardiomyocytes^{9,10}. The repolarizing current modifies the shape and duration of the cardiac action potential. A feature of cardiac hypertrophy has been shown to be the elongation of the cardiac action potential⁹. In this work, we show results that are consistent with the literature; however, it should be noted that although the lengthening of the APD could have occurred as a result of the development of cardiac hypertrophy, the decrease in I_{NBC} could contribute to this phenomenon. Furthermore, the fact that neither a prolongation of the APD nor a depolarization of the RMP were found in the absence of bicarbonate suggests that the responsible for these phenomena is I_{NBC} . Consistent with these results, we show that the effect at the cellular level has a correlation *in vivo* due to the increase in the duration of the QT interval by ECG suggesting an electrophysiological role of I_{NBC} .

In summary, in this work we were able to reduce the expression and activity of NBCe1 in rats through a specific shRNA, which causes an increase in the size of the heart, modifications in the electrical properties and the homeostasis of Ca^{2+} . Increasing knowledge about the role played by each of the NBC isoforms could be of great importance as possible therapeutic targets for treating the cardiac hypertrophy.

Declarations

DATA AVAILABILITY

All data generated or analysed during this study are included in this published article and its supplementary information files.

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Figures

Figure 1

a, Diagram of Adeno-Associated Virus vectors serotype 9 (AAV9) used in this study. eGFP (enhanced green fluorescent protein) is present in both constructs under cytomegalovirus (CMV) promoter. **b**, Scheme of the experimental design. Blood pressure, echocardiography and ECG recordings were obtained before and after 28 days of AAV9 tail injection. On day 28, rats were sacrificed. **c**, Fluorescent images of heart tissue (top) and isolated ventricular myocytes (down) showing eGFP expression. **d**, NBCe1 protein

expression was determined by western-blot. NBCe1 had a significant decrease after 28 days of AAV9-shNBCe1 injection (AAV9-shControl, n= 6 rats; AAV9-shNBCe1, n= 5 rats). NBCe1 activity was determined by a potassium pulse. **e**, representative traces of pH_i recorded from isolated ventricular myocytes from AAV9-shControl rats (n= 5 from 2 rats) and AAV9-shNBCe1 (n= 7 from 3 rats). **f**, average data of the DpH_i. Boxes correspond to upper and lower quartiles, the horizontal line represents the median, and the whiskers mark the minimum and maximum values. Statistical analysis was done by t-Student test after Shapiro-Wilk normality test, p<0.05 was considered significantly different.

Figure 2

a, left ventricular mass index (LVMI) and myocardial wall thickness (**b**) were determined by echocardiographic analysis before (day 0) and after (day 28) tail injection with AAV9-shControl (n= 12) or AAV9-shNBCe1 (n=12). Red dashed lines connect the same animals at time 0 and 28 days. Solid red lines connect the means of each group. **c**, Time course of systolic blood pressure of rats injected with AAV9-shControl (n=12) or AAV9-shNBCe1 (n=12) measured by tail-cuff method [8]. **d** (top) Representative microphotographs of myocytes cross sectional area (CSA) (20X magnification, black bar indicates 10 μm). **d** (bottom), CSA values of rats before 28 days of AAV9-shControl (n= 9 hearts) or AAV9-shNBCe1 (n=8 hearts) injection. 50 cells were measured in at least 10 images obtained from each left ventricle. **e**, mANP levels from shControl (n=5 hearts) and shNBCe1 (n=5 hearts). Boxes correspond to upper and lower quartiles, the horizontal line represents the median, and the whiskers mark the minimum and maximum values. Statistical analysis was done by t-Student test after Shapiro-Wilk normality test, $p < 0.05$ was considered significantly different.

a **b** **c** **Figure 3**

a, Representative action potentials (AP) recordings performed with current-clamp in perforated-patch configuration obtained from isolated ventricular myocytes after transduction with AAV9-shControl or AAV9-shNBCe1. **b**, resting membrane potential (RMP) was significantly depolarized in shNBCe1 compared to shControl. **c**, action potential duration at 50% of repolarization. shNBCe1 ($n=12$ from 5 rats) compared to shControl ($n=12$ from 4 rats). Boxes correspond to upper and lower quartiles, the horizontal line represents the median, and the whiskers mark the minimum and maximum values. Statistical analysis was done by t-Student test after Shapiro-Wilk normality test, $p < 0.05$ was considered significantly different.

Figure 4

a, Representative electrocardiogram (ECG) recordings of rats before (I) and 28 days after (II) AAV9-shControl (n=6) or AAV9-shNBCe1 (n=8) transduction, (III) and (IV). **b**, QT interval corrected by heart frequency (QTc) values from rats after and before the treatment. **c**, Heart frequency remains unaltered after 28 days post AAV injection. Red dashed lines connect the same animals at time 0 and after 28 days. Solid red lines connect the means of each group. Boxes correspond to upper and lower quartiles, the horizontal line represents the median, and the whiskers mark the minimum and maximum values. Statistical analysis was done by t-Student test after Shapiro-Wilk normality test, $p < 0.05$ was considered significantly different.

Figure 5

a, representative western-blot of NBCn1 and NHE1 (**b**) showing the overexpression of both proteins after 28 days of injection with AAV9-shControl (n=6 hearts) and AAV9-shNBCe1 (n=5 hearts). Boxes correspond to upper and lower quartiles, the horizontal line represents the median, and the whiskers mark the minimum and maximum values. Statistical analysis was done by t-Student test after Shapiro-Wilk normality test, $p < 0.05$ was considered significantly different.

Figure 6

a, representative line-scan images of AAV9-shControl or AAV9-shNBCe1 isolated ventricular myocytes paced at 0.5Hz. **b**, values of intracellular calcium transient amplitude showing no differences between shControl (n=10 from 2 rats) or shNBCe1 (n=15 from 4 rats). **c**, typical example of confocal line-scan images of Ca²⁺ wave in isolated ventricular myocytes. **d**, values of Ca²⁺ waves frequency are augmented in rats after 28 days of transduction with AAV9-shNBCe1 (n= 15 myocytes from 2 rats) compared to

AAV9-shControl (n=10 myocytes from 4 rats). Boxes correspond to upper and lower quartiles, the horizontal line represents the median, and the whiskers mark the minimum and maximum values. Statistical analysis was done by Mann-Whitney test after the Shapiro-Wilk normality test, $p < 0.05$ was considered significantly different.

Figure 7

Schematic diagram showing the proposed mechanism involved in the development of heart hypertrophy by the reduction of NBCe1 activity. The ion homeostasis (Na⁺, HCO₃⁻ and H⁺) of the normal heart is carefully controlled in part by the alkalizing mechanisms (NBC and NHE1). However, when there is a modification in the expression of NBCe1 (AAV9-shNBCe1), a rearrangement occurs increasing the expression of NBCn1 and NHE1. This modifies the homeostasis of intracellular Na⁺ which can generate an increase in the concentration of Ca²⁺ and the subsequent development of cardiac hypertrophy.

Supplementary Files

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